OPIC
OFFICE DE LA PROPRIÉTÉ INTELLECTUELLE DU CANADA

CIPO
CANADIAN INTELLECTUAL PROPERTY OFFICE

(12) (19) (CA) **Demande-Application**

(21) (A1) **2,263,848**
(86) 1997/08/21
(87) 1998/02/26

(72) LUBITZ, Werner, AT
(72) JECHLINGER, Wolfgang, AT
(72) SZOSTAK, Michael, AT
(72) WITTE, Angela, AT
(71) EVAX TECHNOLOGIES GMBH VACCINE DEVELOPMENT, DE

(51) Int.Cl.[6] C12N 15/73, C12Q 1/68, C12N 1/21, C12N 15/10, A61K 39/02
(30) 1996/08/21 (196 33 698.8) DE

(54) **NOUVEAUX SYSTEMES DE REGULATION DE L'EXPRESSION GENETIQUE**

(54) **NEW SYSTEMS FOR REGULATING GENETIC EXPRESSION**

(57) La présente invention concerne un procédé pour sélectionner de nouvelles séquences d'opérateurs P_R ou P_L constituées de phages lambdoïdes qui présentent une thermostabilité différente de celle des séquences de type sauvage, pour la fixation d'un répresseur. L'invention concerne en outre de nouvelles séquences d'opérateurs P_R ou P_L mutées, ainsi que leur utilisation pour l'expression génétique thermorégulée et pour la préparation de vaccins améliorés.

(57) The present invention concerns a process for selecting new P_R- or P_L-operator sequences of lambdoid phages which, compared to wild-type sequences, have a different thermostability for the binding of a repressor. In addition, the invention discloses new mutated PR- or PL-operator sequences and their use for temperature-regulated expression of genes and for producing improved vaccines.

New systems for the regulation of gene expression

Description

The present invention concerns a method for selecting new P_R or P_L operator sequences from lambdoid phages which have a different thermostability compared to the wild-type sequence with regard to binding a repressor. In addition new mutated P_R or P_L operator sequences and their application for the temperature-regulated expression of genes and for the production of improved vaccines are disclosed.

The initiation of transcription of the O_R-O_L region of the bacteriophage lambda and other lambdoid phages is negatively and positively regulated by a repressor which is the product of the cI gene (see review article Ptashne et al., Cell 19 (1980), 1-11). In the O_R region three operator sequences (O_R1, O_R2 and O_R3) overlap the promoters P_R and P_{RM} which are orientated in different directions. P_R controls the transcription of genes which are responsible for the lytic multiplication cycle of the phage whereas P_{RM} is the promoter for the lambda cI gene which is responsible for maintaining the lysogenic state. The lambda repressor cI binds co-operatively to the operator sequences O_R1 and O_R2 with the result that P_R is repressed and P_{RM} is activated.

In addition the bacteriophage lambda also contains a further operator region O_L which also contains three operator sequences (O_L1, O_L2 and O_L3). The expression of the lambda N gene can be repressed by the P_L promoter by binding of the cI repressor to this O_L operator region.

Promoters of the bacteriophage lambda in particular the P_L and the P_R promoter have been used for a long time in recombinant DNA technology for heterologous temperature-regulated gene expression in E.coli (cf. Hedgpeth et al., Molec.Gen.Genet. 183 (1978), 197-203 and Bernard et al., Gene 5 (1979), 59-76; Buell et al., Nucleic Acids Res. 13 (1985), 1923 and Shatzman and Rosenberg, Methods Enzymol. 152 (1987), 661). A temperature-sensitive lambda repressor cI857 is used in these expression systems which represses the P_L and P_R transcription at low temperatures up to 30°C but allows a gene expression at higher temperatures.

An advantage of this lambda expression system is that the gene expression can be induced in a simple manner by increasing the temperature and no addition of chemical inducers is necessary for this. However, a serious disadvantage is that the repression of gene expression only occurs up to relatively low temperatures of not more than 30°C, which is a temperature at which only a slow bacterial growth occurs. Hence the object of the invention was to provide an improved system for lambda P_L or P_R gene expression which enables a repression at variable higher temperatures.

This object is achieved by providing mutated P_R or P_L operator sequences from lambdoid phages which, compared to the wild-type operator sequence, have a different and in particular higher thermostability with regard to the binding of a temperature-sensitive repressor. The finding that lambda expression systems with an improved thermostability can be produced at all is extremely surprising since, apart from the temperature-sensitive lambda cI857 mutant, no other temperature-sensitive cI mutants are known but only those mutations in the cI

repressor are known which make the molecule more resistant to thermal inactivation (Hecht et al., Proteins 1 (1986), 43-46 and Das and Mandal, Mol.Gen.Genet. 204 (1986), 540-542). It was even more surprising that mutations which lead to an improved thermostability are located in the operator DNA sequence and not in the DNA sequence coding for the repressor molecule. Thus for example a mutation of the lambda O_R2 operator sequence is known from the literature which leads to a complete loss of repressor binding (Hawley et al., J.Biol.Chem. 260 (1985), 8618-8626).

A method is provided for identifying suitable mutants which enables the selection of mutated O_R or O_L operator DNA sequences from lambdoid phages which have a different thermostability compared to the wild-type sequence with regard to binding a repressor in which the method is characterized in that (a) a DNA cassette is prepared which contains a selection gene under the operative control of an expression control sequence comprising at least one O_R or O_L operator sequence from a lambdoid phage and a promoter, (b) the operator DNA sequence is subjected to a mutagenesis and (c) the mutated operator DNA sequences are analysed.

The lambdoid phages are preferably selected from the group comprising the phage lambda, phage 21, phage 22, phage 82, phage 424, phage 434, phage D326, phage DLP12, phage gamma, phage HK022, phage P4, phage Phi80, phage Phi81, coliphage 186 and recombinant variants thereof. The said phages are very similar with regard to the mechanism of repression of gene expression by means of a cI repressor (Johnson et al., Nature 294 (1982), 217-223). Recombinant variants of the said phages e.g. lambda imm434 can be obtained by substitution of

individual genome fragments within the said phages (cf. for this Hendricks et al., Lambda 2 (1983), R.W. Hendricks, J.W. Roberts, F.W. Stahl and R.A. Weissberg (publisher), Cold Spring Harbor Laboratory Press, New York). The phage lambda or a recombinant variant thereof is preferably used as the lambdoid phage e.g. lambda imm434. An operator DNA sequence from the operator regions O_R (SEQ ID NO.1) or /and O_L (SEQ ID NO.3) of the phage lambda and in particular one of the operator sequences O_R1, O_R2 and O_R3 or O_L1, O_L2 and O_L3 contained therein is particularly preferably used for the mutagenesis. The operator sequence O_R2 is most preferred.

The selection gene for the DNA cassette which is brought under the operative control of the expression control sequence containing the mutated operator sequence, preferably a lambda operator/promoter region, is preferably a suicide gene which when expressed leads to the death of the bacterial cell and thus serves as a selection marker for identifying suitable mutants. The suicide gene should be so strongly repressed at a temperature at which the lambda repressor binds to the mutated operator sequence that a bacterial cell containing the DNA cassette can grow. When the maximum temperature at which the repressor can still bind to the operator is exceeded, the suicide gene is expressed and the bacterial cell is destroyed. This enables a simple and direct selection of suitable mutated operator sequences. A suitable suicide gene is the E lysis gene from the phage PhiX174 as well as homologues and derivatives derived therefrom (Hutchison and Sinsheimer, J.Mol.Biol. 18 (1966), 429-447; Witte et al., Multifunctional safety vector systems for DNA cloning, controlled expression of fusion genes and

simplified preparation of vector DNA and recombinant gene products, in BioTech Forum, Advances in Molecular Genetics 3, pp 219-239, publisher: Issinger, O.-G., Henke, J., Kämpf, J., Driesel, A.J., Hüthing Verlag 1991, Heidelberg). Further examples of suitable lysis genes are GEF (Poulsen et al., Mol.Microbiol. 5 (1991), 1627-1637) and Kil (Reisinger et al., Virology 193 (1993), 1033-1036). On the other hand the selection gene can also be a reporter gene such as e.g. the ß-Gal gene.

In order to produce mutants the operator DNA sequence is preferably subjected to a site-specific mutagenesis using one or several oligonucleotides for example according to the method of Kunkel (Proc.Natl.Acad.Sci. USA 82 (1985), 488-492) or they are obtained by selection in a mutator bacterial strain e.g. an E. coli mutD or mutL mutator strain such as E. coli ES1578 (Wu et al., Gene 87 (1990), 1-5). The mutated operator DNA sequences are preferably selected by determining the ability to bind to a temperature-sensitive cI repressor in particular to the temperature-sensitive cI857 repressor. For this the DNA cassette which is preferably located on a vector is transformed into a bacterial cell which contains a gene coding for a temperature-sensitive cI repressor. This gene may also be present on a vector (Remaut et al., Gene 15 (1981), 81-93). On the other hand it is possible to use a bacterial cell which contains such a repressor gene in its chromosome e.g. E. coli M5219 (cf. e.g. Shimatake and Rosenberg, Nature 292 (1981), 128).

Mutants which are resistant to lysis at different temperatures can be identified in a simple manner by culturing the bacterial cells transformed with a lysis cassette which contain the mutated operator DNA

sequences. Up to now it has been possible to identify several mutants which are resistant to a lysis at temperatures up to 33°C, 35°C, 37°C and 39°C. These bacteria contain mutated operator DNA sequences which allow binding of the repressor up to the respective temperature. A particularly preferred example is a mutant to which the cI857 repressor binds up to a temperature of about 37°C. Compared to the wild-type the mutation is a single base substitution in the O_R2 section of the lambda O_R operator region. The sequence of this mutated lambda O_R operator is shown in SEQ ID NO.2.

An additional subject matter of the invention are mutated O_R or O_L operator sequences from lambdoid phages which have a different thermostability compared to the wild-type sequence with regard to binding of a repressor and which are obtainable by the selection methods described above. The mutated O_R or O_L operator sequences preferably have an increased thermostability with regard to the binding of a temperature-sensitive repressor and in particular of the temperature-sensitive cI repressor. The mutated operator sequences particularly preferably have a thermostability that is increased by about 3 to 10°C, in particular by about 7 to 9°C compared to the wild-type sequence.

Since the selection method according to the invention is preferably carried out on O_R or O_L operator sequences which are derived from the phage lambda, the present invention in particular concerns mutated lambda O_R or O_L operator sequences which are variants of the O_R operator sequences shown in SEQ ID NO.1 or variants of the O_L operator sequences shown in SEQ ID NO.3. Variant in this connection is understood as an operator sequence which differs from the wild-type sequence in at least one

sequence position by insertion, deletion or substitution of bases. The differences are particularly preferably in the region of the sections O_R1, O_R2 or O_R3 and O_L1, O_L2 and O_L3. A specific example of a mutated lambda operator sequence according to the invention is the lambda-O_R operator sequence shown in SEQ ID NO.2.

The mutated operator sequences allow the production of new temperature-regulated systems for gene expression in which microorganisms and in particular bacteria can be cultured in a repressed state at variable temperatures and preferably at higher temperatures than have been previously possible in particular at 33 to 39°C. Hence a subject matter of the invention is the use of the mutated O_R or O_L operator sequences for the temperature-regulated expression of genes in bacteria and in particular in gram-negative bacteria such as E. coli. Combination of a wild-type O_R or O_L operator region and at least one operator region which contains a mutated operator sequence according to the invention or combination of several operator regions which contain mutated operator sequences according to the invention with different thermostabilities even enables a temperature-regulated sequential expression of genes.

Vectors and bacterial strains in which the inventive mutated operator sequences can be used for the temperature-regulated expression of genes are familiar to a person skilled in the art. In this case the expression systems known from the prior art containing the lambda cI857 repressor in combination with a suitable promoter e.g. the lambda P_L or lambda P_R promoter can be used (cf. e.g. Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd Edition, 1989, Cold Spring Harbor Laboratory Press, New York,

17.11-17.12).

A further subject matter of the present invention is a nucleic acid comprising a bacterial expression control sequence i.e. a sequence containing a promoter and operator regions which contains a mutated O_R or O_L operator sequence according to the invention in operative linkage with a protein-coding sequence. The protein-coding sequence can for example be a sequence coding for a eukaryotic protein or polypeptide or a bacterial gene e.g. the E-lysis gene.

An additional subject matter of the present invention is a vector which contains at least one copy of the bacterial expression control sequence in operative linkage with the protein-coding sequence. This vector can be any prokaryotic vector e.g. a chromosomal vector such as a bacteriophage or an extrachromosomal vector such as a plasmid. Suitable prokaryotic vectors are described for example by Sambrook et al., Supra, chapters 1-4.

Yet a further subject matter of the present invention is a bacterial cell which is transformed with a nucleic acid according to the invention or with a vector according to the invention. In a preferred embodiment the cell is a gram-negative prokaryotic cell, particularly preferably an E. coli cell. The cell preferably contains the nucleic acid or the vector integrated into its chromosome and in addition contains a gene for a cI repressor from a lambdoid phage in particular the gene for the lambda cI857 repressor.

A particularly preferred application of the mutated

operators according to the invention is in the field of vaccine production. So-called "bacterial ghosts" are known as vaccines from the prior art i.e. bacterial coats that can be prepared from gram-negative bacteria such as E. coli, Salmonella typhimurium, Klebsiella pneumoniae, Actinobacillus pleuropneumoniae etc. by means of protein-E-induced lysis. These ghosts whose cell surface properties and repertoire of surface antigens that can be recognized by the immune system are very similar to the active pathogen, produce a protective cellular or/and humoral immune response in various animal models.

The process for preparing the ghosts is based on the stringent controlled expression of the E-lysis gene from PhiX174 whose expression product forms a tunnel through the bacterial cell wall coat and thus leads to a pouring out of the cell contents of the host cell. This lethal gene for the cells can be regulated by means of a lambda repressor e.g. the temperature-sensitive lambda repressor cI857 which, as described above, loses its function at temperatures above 30°C. As a result, the bacterial cultures that have previously been used to produce bacterial ghosts have had to be cultured at low temperatures, preferably at 28°C.

Although this method leads to satisfactory results with regard to the immunogenicity of the ghosts that are produced, an improvement of the bacterial culture is urgently required since the repertoire of antigenic determinants on the bacterial surface can change depending on the external conditions. Since pathogenic bacteria which infect humans or animals usually colonize at an environmental temperature of 37 to 39°C, this natural environmental temperature should also be

maintained during the production process for the ghosts.

A process for producing bacterial ghosts which achieves this object is provided by using the mutated operator sequences according to the invention. These operator sequences allow growth of the bacteria up to a temperature range of preferably 35 to 39°C and allow lysis when the temperature is increased from 37 to 42°C. This changed lysis behaviour enables the pathogens to be cultured near to the body temperature of the vaccine candidate which is extremely important for the composition of the external membrane. Furthermore the new lysis cassette can also be used as a safety cassette in live vaccines since for example in humans the inoculated bacteria are killed when fever is induced (39°C).

Hence a subject matter of the invention is a vaccine composition which contains a live bacterial cell according to the invention as the active ingredient optionally together with pharmaceutically tolerated auxiliary substances, additives and carrier substances. The live bacterial cell contains a nucleic acid comprising a bacterial expression control sequence with a mutated operator sequence in operative linkage preferably with a lysis gene. Yet a further subject matter of the present invention is a vaccine composition which contains a bacterial ghost as an active ingredient optionally together with pharmaceutically tolerated auxiliary substances, additives and carrier substances wherein the bacterial ghost is obtainable by culturing a bacterial cell according to the invention at temperatures of 35 - 39°C and subsequently lysing the bacterial cell by increasing the temperature. Bacterial cells suitable as vaccines are in particular gram-

negative bacteria such as E. coli for example the strains STEC, EHEC, O78:K80, Salmonellae such as S.choleraesuis, S.enteritidis and S.typhimurium, Pasteurella multocida, Pasteurella haemolytica, Bordetella bronchiseptica, Klebsiella pneumoniae, Actinobacillus pleuropneumoniae, Haemophilus influenzae, Vibrio cholerae, Helicobacter pylori, Alcaligenes eutrophus, Campylobacter jejuni and Pseudomonas aeruginosa.

The vaccine compositions modified according to the invention can be transferred orally, aerogenically or parenterally to the vaccine candidates. The route which the corresponding microorganisms naturally select for the infection and for the initial stages of establishing an infectious disease are preferably selected for the application of the vaccine. Since all surface properties are retained in the vaccines according to the invention, this application can result in a local induction of the immune response as also occurs in the natural infection process.

As described above the use of mutated operator sequences according to the invention enables the development of vaccines that can be lysed in a controlled manner when a target temperature is exceeded. Furthermore it is, however, also possible to provide a cold-sensitive suicide cassette which on release into the environment kills gram-negative bacteria that are used as a live vaccine. Hence combination of two genetic regulation systems enables the bacteria to die as a result of the expression of a suicide gene when a target value for the environmental temperature is exceeded. This safety cassette ensures that the live vaccines are killed even if they are eliminated from the organisms.

Hence the invention concerns a nucleic acid comprising (a) a first bacterial expression control sequence which contains an O_R or O_L operator sequence from a lambdoid phage and to which a first temperature-sensitive cI repressor from lambdoid phages can bind in operative linkage with a sequence coding for a second repressor wherein the second repressor cannot bind to the first bacterial expression sequence and (b) a second bacterial expression control sequence to which the second repressor can bind which is in operative linkage with a suicide gene.

The components (a) and (b) can be covalently linked together e.g. be present on a single vector or be separate from one another e.g. present on different vectors or be located separately or together on the chromosome of a recipient bacterium.

Yet a further subject matter of the present invention is a bacterial cell which contains at least one copy of a nucleic acid as described above. In addition the bacterial cell advantageously contains a gene for the first repressor. The first repressor is preferably the temperature-sensitive cI857 repressor.

The safety cassette according to the invention preferably contains a gene which codes for a temperature-sensitive cI repressor e.g. the repressor cI857 and a gene which codes for a second repressor wherein this gene is under the control of a lambda promoter/operator region to which the temperature-sensitive repressor binds. The second repressor in turn controls the expression of another gene e.g. a suicide gene such as the E-lysis gene. The temperature-sensitive

lambda repressor is inactive at 37°C so that the second repressor is expressed which in turn represses the expression of the suicide gene.

When the temperature is reduced the temperature-sensitive lambda repressor binds to the operator so that the expression of the second repressor is blocked which leads to an expression of the suicide gene. A first expression control sequence is preferred for this safety cassette which contains the mutated lambda operator since this enables an improved and more rapid activation of the suicide gene.

The second repressor can be any repressor e.g. a lac repressor. However, it is preferable to use an additional repressor from lambdoid phages e.g. cI from the phage 434 which is not temperature-sensitive and binds to its own operator sequence but does not bind to the sequence recognized by the lambda repressor cI857.

It is particularly preferable for the development of live vaccines to incorporate a heat as well as a cold regulation element. This incorporation is preferably achieved by homologous recombination into the chromosome of the vaccine bacterium.

Thus the present invention also concerns a bacterial cell which, in addition to the two components (a) and (b), contains a third bacterial expression control sequence as component (c) which contains a mutated operator sequence according to the invention in operative linkage with a suicide gene.

These bacterial cells can also be used in vaccine compositions and especially for live vaccines. In this manner it is possible to produce heat or/and cold-sensitive safe live vaccines which lead to death of the vaccine bacteria when the body temperature of the vaccine candidate is increased e.g. by fever or/and when they are excreted into the environment.

It is intended to additionally elucidate the invention by the following figures, sequence protocols and examples.

Fig. 1a shows a schematic representation of a lysis cassette of the prior art comprising a lambda O_R wild-type region, the lambda cI857 gene under the control of the promoter P_{RM} and the E lysis gene under the control of the promoter P_R;

Fig. 1b shows a schematic representation of a lysis cassette according to the invention which contains a mutated lambda O_R sequence;

Fig. 2a shows a schematic representation of a cold-sensitive safety cassette comprising a wild-type (pCS1) or mutated (pCSJ1) O_R operator sequence, the lambda-cI857 gene under the control of the promoter P_{RM}, the gene of the lacI repressor under the control of P_R and the E-lysis gene under the control of the lac promoter/operator system at a temperature at which the temperature-sensitive lambda repressor cI857 does not bind to the lambda O_R sequence;

Fig. 2b shows a schematic representation of the safety cassette according to Fig. 2a at a temperature at which the lambda repressor cI857 binds to the lambda O_R operator;

Fig. 3 shows the lysis curve of bacterial cells (optical density versus time) which contain a plasmid with the lysis cassette shown in Fig. 1b;

Fig. 4 shows the lysis curve of a bacterial cell which contains a cold-sensitive safety cassette with the wild-type O_R operator and

Fig. 5 shows a comparison of lysis curves of bacterial cells which contain a cold-sensitive safety lysis cassette with the wild-type O_R operator (pSC1) or the mutated operator (pCSJ1);

Fig. 6a shows a schematic representation of a cold-sensitive safety cassette comprising a wild-type (pCS2) or mutated (pCSJ2) O_R operator sequence, the lambda cI857 gene under the control of the promoter P_{RM}, the gene of the phage 434 cI repressor under the control of lambda P_R and the E lysis gene under the control of the 434 O_R (P_{RM}-P_R) promoter/operator system at a temperature at which the temperature-sensitive lambda repressor cI857 does not bind to the lambda O_R sequence,

Fig. 6b shows a schematic representation of the safety cassette according to Fig. 6a at a temperature at which the lambda repressor cI857 binds to the lambda O_R operator;

SEQ ID NO.1 shows the nucleotide sequence of the lambda O_R operator; the operator sequence O_R3 extends from position 11 - 27; the operator sequence O_R2 extends from position 34 - 41; the operator sequence O_R1 extends from position 58 - 74;

SEQ ID NO.2 shows the nucleotide sequence of a mutated lambda O_R operator which, compared to the wild-type sequence, has a substitution of

T → C at position 42;

SEQ ID NO.3 shows the nucleotide sequence of the lambda O_L operator; the operator sequence O_L3 extends from position 11 - 27; the operator sequence O_L2 extends from position 31 - 47; the operator sequence O_L1 extends from position 55 - 70:

SEQ ID NO. 4 to 6

show a 1601 bp long DNA fragment of the plasmid pAW12; bp 1 - 983 are derived from the bacteriophage lambda (position 37125 - 38107; cf. Sanger et al., J.Mol.Biol. 162 (1982), 729-773) and contain the lambda cI857 gene (position 816-106; SEQ ID NO.5) as well as the mutated O_R operator region (mutation at position 858 T → C); bp 1023 - 1601 are derived from the phage PhiX174 (position 447 - 1026; cf. Sanger et al., J.Mol.Biol. 125 (1978), 225-246) and contain the E-lysis gene (position 1144 - 1416; SEQ ID NO.6);

SEQ ID NO. 7 to 10

shows a 2834 bp long DNA fragment of the plasmid pCSJ; bp 1 - 983 are derived from the bacteriophage lambda (position 37125 - 38107) and contain the cI857 gene (position 816 - 106; SEQ ID NO.5) as well as the mutated lambda O_R region (mutation at position 858 T → C; bp 990 - 2230 are derived from the E. coli lac operon subcloned on the plasmid pMC7 (Calos, Nature 274 (1978), 762-765) and contain the lacI repressor gene (bp 1025 - 2104; SEQ ID NO.9) and the lac promoter/operator; bp 2256 - 2834 are derived from the bacteriophage

PhiX174 (position 447 - 1026) and contain the E-lysis gene (bp 2377 - 2649; SEQ ID NO.10).

Examples

Example 1:

1.1 Random mutagenesis of the lambda O_R operator region

The plasmid pAW12 (Witte and Lubitz, Eur.J.Biochem. 180 (1989), 393-398) was selected as the starting material which contains the lysis gene E from the bacteriophage PhiX174 under the control of the lambda P_R promoter as well as the associated repressor gene cI857. The aim of this experiment was to change the lysis cassette so that the lysis gene E is not already activated at 30°C but at higher temperatures. For this the E. coli mutator strain ES1578 (Wu et al., (1990), supra) was transformed with the lysis plasmid and a selection was carried out for clones with a changed temperature profile of cell lysis.

For this the mutated clones produced by the transformation were detected after being stamped onto test plates containing lysis selective medium (LB containing 1 % SDS) and incubated at different temperatures (e.g. 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C). The changed lysis profile of the lysis cassette in liquid culture was exactly determined by plasmid extraction and subsequent transformation into a non-mutator test strain.

The type of mutation was determined by subcloning the

mutagenized lysis cassettes into a sequencing plasmid. In addition the lysis gene E was substituted by the β-galactosidase gene for a functional check. It was then possible on the basis of a simple β-gal test to quantitatively measure the repressed or active state of the gene cassette.

In this manner is was possible to obtain several clones with a different temperature lysis profile. These clones allowed growth of the bacteria in a temperature range of 33-39°C and did not lead to lysis of the bacteria until the temperature was further increased to 37-42°C.

A mutation of the O_R operator region (SEQ ID NO.2) was identified by sequencing a mutated clone which had a thermostability up to 37°C.

1.2 Verification of the mutation

In order to verify the mutation obtained in example 1.1. a site-specific mutagenesis of the lambda O_R wild-type sequence was carried out using an oligonucleotide.

The mutagenesis was carried out according to the protocol of Kunkel (Proc.Natl.Acad.Sci. USA 82 (1985), 488-492).

4 ml overnight culture of the E. coli strain CJ236 (dut^-, ung^-) was added to 50 ml LB medium (+ 10 µg/ml chloramphenicol and 0.25 µg/ml uridine) and shaken for 30 min at 37°C. Then 100 µl M13 phages was added and it was incubated for 6 h at 37°C.

The culture was centrifuged in 2 SS34 centrifuge tubes for 10 min at 14000 rpm and 4°C, the supernatant was decanted into new tubes and again centrifuged for further purification.

The phages were precipitated for 1 h at 4°C by addition of 5 ml 5 x polyethylene glycol/NaCl. They were then centrifuged for 10 min at 14000 rpm and 4°C and the supernatant was discarded.

The pellet was dried, suspended in 0.8 ml TES buffer (0.1 M Tris HCl, pH 8; 0.3 M NaCl; 1 mM EDTA) and incubated for 1 h at 4°C. The suspension was divided into 2 Eppendorf vessels and centrifuged for 5 min at 5000 rpm. The supernatant in which the disrupted phages were located was removed and subjected to a phenol/chloroform extraction to isolate the DNA. The resulting DNA was precipitated with a 2.5-fold volume of 96 % ethanol, washed with 70 % ethanol and taken up in 60 µl H_2O.

An oligonucleotide with the sequence
5'-GTA AAA TAG TCA ACA CGC GCG GTG TTA GAT ATT TAT C-3'
was phosphorylated. For this 20 µl H_2O, 20 µl oligonucleotide (20 ng), 4.5 µl kinase buffer (Stratagene) and 0.5 µl polynucleotide kinase (5 U, Stratagene) was incubated for 1 h at 37°C. Then 7 µl 0.1 M EDTA was added and it was heated for 10 min to 65°C.

For the annealing 20 µl phosphorylated oligonucleotide, 3.5 µl single-stranded DNA template (1 µg ssDNA produced as described above) and 1.4 µl 20 x SSC buffer were heated for 5 min to 70°C, slowly cooled to 25°C and then

placed on ice.

For the extension 10 µl of the reaction mixture from the annealing mixture, 37.5 µl XL buffer (27 mM Hepes pH 7.8, 5 mM of each dNTP, 13 mM $MgCl_2$, 2.7 mM dithiothreitol, 1.3 mM ATP, 1 µl ligase (1 U, Boehringer Mannheim), 1.5 µl T4 polymerase (1.5 U, Boehringer Mannheim), 1.5 µl T4 gene32 protein (8 µg, Boehringer Mannheim) were incubated for 10 min on ice, 10 min at room temperature and 2 h at 37°C. After 1 h 1 µl ligase and 1 µl T4 DNA polymerase was added. After completion of the incubation the reaction was stopped by adding 3 µl 0.25 M EDTA.

For the transformation 100 µl competent E. coli cells JM103 (Messing et al., Nucleic Acids Res.9 (1981), 309-321) was admixed with 10 µl DNA from the extension mixture and incubated for 1 h or more on ice. After a heat shock for 2.5 min at 42°C, 0.2 ml fresh JM103 cells was added in the logarithmic growth phase. The cells were mixed with 3 ml soft agar and inoculated on an LB agar plate. They were subsequently incubated overnight at 37°C.

In order to identify the mutants, plaques were pricked out with a Pasteur pipette and used to inoculate 5 ml LB medium to which 400 µl of an overnight culture of E. coli JM103 had been added. After 3 h growth at 37°C, the cells were centrifuged. Double-stranded M13 plasmids were obtained from the cell pellet by means of plasmid preparation. Single-stranded M13 phages could be isolated from the supernatant.

Example 2:

Analysis of the mutagenized lysis cassettes

Figures 1 and 2 shows different E-specific lysis cassettes with different temperature inductions of the lysis function.

In Fig. 1a which contains the wild-type lambda O_R operator sequence, the function of the E-lysis gene is repressed up to 30°C by the cI857-coded repressor protein on the preceding lambda P_R promoter/operator region. cI857-specific repressor molecules are thermally inactivated at temperatures above 30°C and the expression of the E gene is induced. Fig. 1b shows the plasmid pAWJ12 which contains a mutated operator sequence (SEQ ID NO.2) so that the repression of the lysis function of the gene E by cI857 occurs up to 37°C and the lysis function is not induced until 39°C or higher temperatures are reached.

The function of a cold-sensitive safety cassette is elucidated in Fig. 2. Fig. 2a shows that the formation of lacI-specific repressor molecules which in turn repress the expression of the E gene is induced in the plasmids pCS1 (wild-type operator) and pCSJ1 (mutated operator) at a temperature of $\geq$ 32°C (pCS1) or $\geq$ 39°C (pCSJ1). At a temperature below 30°C (pCS1) or 37°C (pCSJ1) functional cI857 repressor molecules are formed which suppress the formation of lacI-specific repressor molecules and thus allow the expression of the E gene (Fig. 2b). In the plasmid pCSJ1 the concomitant cell lysis occurs more rapidly than in pCS1.

Fig. 3 shows the lysis curve of a bacterial cell containing the plasmid pAWJ12 (mutated operator). 3 hours after beginning the culture, the temperature was maintained at 37°C in an aliquot of the bacterial cells and increased in two other aliquots to 38 and 42°C. At 37°C there was a further growth of the bacteria whereas a lysis already occurred at 38°C. The lysis is considerably increased at 42°C.

Figures 4 and 5 show the function of a cold-sensitive safety cassette. In Fig. 4 bacterial cells which contained the plasmid pCS1 (wild-type operator) were subjected to a temperature change from 37 to 28°C. This reduction in temperature led to the E-lysis gene being switched off and cell death (decrease of the optical density).

Fig. 5 shows a comparison of the lysis rate of bacteria which contain the plasmid pCS1 (wild-type operator) and the plasmid pCSJ1 (mutated operator). It can be seen that the lysis occurs much more rapidly in the bacteria which contain the mutated operator.

Fig. 6 shows a further cold-sensitive safety cassette. At temperatures at which the lambda cI857 repressor does not bind to the operator the plasmids pCS2 (wild-type operator) and pCSJ2 (mutated operator) form cI-434 repressor molecules which repress the expression of the E gene (Fig. 6a). Formation of cI-434-specific repressor molecules is prevented thus allowing expression of the E gene at a temperature at which the cI857 repressor binds to the lambda operator (Fig. 6b).

Example 3:

In vivo analysis of cold-sensitive lysis cassettes

The killing of bacteria by lowering the temperature after passage through a mouse intestine and excretion into the faeces was determined.

For this 10^{10} E. coli bacteria were administered once to Balb/c mice and the excreted number of bacteria in the faeces was determined. The evaluation was carried out on E.coli-specific Endo plates (Endo, "Zentralbl. Bakt. I Orig." 35 (1904) 109-110) using tetracyclin as a marker for the plasmids used. The incubation was carried out at 28°C.

Results:

In the experimental groups E. coli NM522 (pCS2), E. coli MC4100 (pCS1) and E. coli MC4100 (pCSJ1) there was a reduction in the germ count compared to an E. coli NM522 (pAWJ-lac) control of at least 99.9 %, 98 % and 80 % measured 10 h and 20 h after administering the E. coli bacteria.

SEQUENCE PROTOCOL

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Prof.DR. Werner Lubitz
(B) ROAD: Schoenborngasse 12/7
(C) CITY: Vienna
(E) COUNTRY: Austria
(F) POSTAL CODE: 1080

(ii) TITLE OF INVENTION: New systems for the regulation of gene expression

(iii) NUMBER OF SEQUENCES: 10

(iv) COMPUTER-READABLE FORM:
(A) DATA CARRIER: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 82 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(vi) INITIAL ORIGIN:
(A) ORGANISM: lambda OR operator (wild-type)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ACGTTAAATC TATCACCGCA AGGGATAAAT ATCTAACACC GTGCGTGTTG ACTATTTTAC 60

CTCTGGCGGT GATAATGGTT GC 82

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 82 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(vi) INITIAL ORIGIN:
(A) ORGANISM: lambda OR operator (mutant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ACGTTAAATC TATCACCGCA AGGGATAAAT ATCTAACACC GCGCGTGTTG ACTATTTTAC 60

CTCTGGCGGT GATAATGGTT GC 82

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 85 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(vi) INITIAL ORIGIN:
(A) ORGANISM: lambda OL operator (wild-type)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ACATACAGAT AACCATCTGC GGTGATAAAT TATCTCTGGC GGTGTTGACA TAAATACCAC 60

TGGCGGTGAT ACTGAGCACA TCAGC 85

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1601 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: double strand
(D) TOPOLOGY: both

(vi) INITIAL ORIGIN:
(A) ORGANISM: pAW12 fragment

(xi) CHARACTERISTICS:
(A) NAME/KEY: CDS
(B) LOCATION: complement (106..816)

(ix) CHARACTERISTICS:
(A) NAME/KEY: CDS
(B) LOCATION: 1144..1416

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
ATTTACTATG TTATGTTCTG AGGGGAGTGA AAATTCCCCT AATTCGATGA AGATTCTTGC      60
TCAATTGTTA TCAGCTATGC GCCGACCAGA ACACCTTGCC GATCAGCCAA ACGTCTCTTC     120
AGGCCACTGA CTAGCGATAA CTTTCCCCAC AACGGAACAA CTCTCATTGC ATGGGATCAT     180
TGGGTACTGT GGGTTTAGTG GTTGTAAAAA CACCTGACCG CTATCCCTGA TCAGTTTCTT     240
GAAGGTAAAC TCATCACCCC CAAGTCTGGC TATGCAGAAA TCACCTGGCT CAACAGCCTG     300
CTCAGGGTCA ACGAGAATTA ACATTCCGTC AGGAAAGCTT GGCTTGGAGC CTGTTGGTGC     360
GGTCATGGAA TTACCTTCAA CCTCAAGCCA GAATGCAGAA TCACTGGCTT TTTTGGTTGT     420
GCTTACCCAT CTCTCCGCAT CACCTTTGGT AAAGGTTCTA AGCTTAGGTG AGAACATCCC     480
TGCCTGAACA TGAGAAAAAA CAGGGTACTC ATACTCACTT CTAAGTGACG GCTGCATACT     540
AACCGCTTCA TACATCTCGT AGATTTCTCT GGCGATTGAA GGGCTAAATT CTTCAACGCT     600
AACTTTGAGA ATTTTTGTAA GCAATGCGGC GTTATAAGCA TTTAATGCAT TGATGCCATT     660
AAATAAAGCA CCAACGCCTG ACTGCCCCAT CCCCATCTTG TCTGCGACAG ATTCCTGGGA     720
TAAGCCAAGT TCATTTTTCT TTTTTTCATA AATTGCTTTA AGGCGACGTG CGTCCTCAAG     780
CTGCTCTTGT GTTAATGGTT TCTTTTTTGT GCTCATACGT TAAATCTATC ACCGCAAGGG     840
ATAAATATCT AACACCGCGC GTGTTGACTA TTTTACCTCT GGCGGTGATA ATGGTTGCAT     900
GTACTAAGTA GGTTGTATGG AACAACGCAT AACCCTGAAA GATTATGCAA TGCGCTTTGG     960
GCAAACCAAG ACAGCTAAAG ATCCTCTAGA GTCGACCTGC AGGCATGCAA GCTTATCGAA    1020
TTCTCATTCA GGCTTCTGCC GTTTTGGATT TAACCGAAGA TGATTTCGAT TTTCTGACGA    1080
GTAACAAAGT TTGGATTGCT ACTGACCGCT CTCGTGCTCG TCGCTGCGTT GAGGCTTGCG    1140

TTT ATG GTA CGC TGG ACT TTG TGG GAT ACC CTC GCT TTC CTG CTC CTG      1188
    Met Val Arg Trp Thr Leu Trp Asp Thr Leu Ala Phe Leu Leu Leu
      1               5                  10                  15

TTG AGT TTA TTG CTG CCG TCA TTG CTT ATT ATG TTC ATC CCG TCA ACA      1236
Leu Ser Leu Leu Leu Pro Ser Leu Leu Ile Met Phe Ile Pro Ser Thr
                  20                  25                  30

TTC AAA CGG CCT GTC TCA TCA TGG AAG GCG CTG AAT TTA CGG AAA ACA      1284
Phe Lys Arg Pro Val Ser Ser Trp Lys Ala Leu Asn Leu Arg Lys Thr
              35                  40                  45
```

```
TTA TTA ATG GCG TCG AGC GTC CGG TTA AAG CCG CTG AAT TGT TCG CGT      1332
Leu Leu Met Ala Ser Ser Val Arg Leu Lys Pro Leu Asn Cys Ser Arg
          50                  55                  60

TTA CCT TGC GTG TAC GCG CAG GAA ACA CTG ACG TTC TTA CTG ACG CAG      1380
Leu Pro Cys Val Tyr Ala Gln Glu Thr Leu Thr Phe Leu Leu Thr Gln
      65                  70                  75

AAG AAA ACG TGC GTC AAA AAT TAC GTG CAG AAG GAG TGATGTAATG           1426
Lys Lys Thr Cys Val Lys Asn Tyr Val Gln Lys Glu
 80                  85                  90

TCTAAAGGTA AAAAACGTTC TGGCGCTCGC CCTGGTCGTC CGCAGCCGTT GCGAGGTACT    1486

AAAGGCAAGC GTAAAGGCGC TCGTCTTTGG TATGTAGGTG GTCAACAATT TTAATTGCAG    1546

GGGCTTCGGC CCTTACTTGA GGATAAATTA TGTCTAATAT TCAAACTGGC GCCGA         1601
```

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 237 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
Met Ser Thr Lys Lys Lys Pro Leu Thr Gln Glu Gln Leu Glu Asp Ala
 1               5                  10                  15

Arg Arg Leu Lys Ala Ile Tyr Glu Lys Lys Lys Asn Glu Leu Gly Leu
            20                  25                  30

Ser Gln Glu Ser Val Ala Asp Lys Met Gly Met Gly Gln Ser Gly Val
        35                  40                  45

Gly Ala Leu Phe Asn Gly Ile Asn Ala Leu Asn Ala Tyr Asn Ala Ala
    50                  55                  60

Leu Leu Thr Lys Ile Leu Lys Val Ser Val Glu Glu Phe Ser Pro Ser
65                  70                  75                  80

Ile Ala Arg Glu Ile Tyr Glu Met Tyr Glu Ala Val Ser Met Gln Pro
                85                  90                  95

Ser Leu Arg Ser Glu Tyr Glu Tyr Pro Val Phe Ser His Val Gln Ala
            100                 105                 110

Gly Met Phe Ser Pro Lys Leu Arg Thr Phe Thr Lys Gly Asp Ala Glu
        115                 120                 125

Arg Trp Val Ser Thr Thr Lys Lys Ala Ser Asp Ser Ala Phe Trp Leu
    130                 135                 140

Glu Val Glu Gly Asn Ser Met Thr Ala Pro Thr Gly Ser Lys Pro Ser
145                 150                 155                 160
```

Phe Pro Asp Gly Met Leu Ile Leu Val Asp Pro Glu Gln Ala Val Glu
165 170 175

Pro Gly Asp Phe Cys Ile Ala Arg Leu Gly Gly Asp Glu Phe Thr Phe
180 185 190

Lys Lys Leu Ile Arg Asp Ser Gly Gln Val Phe Leu Gln Pro Leu Asn
195 200 205

Pro Gln Tyr Pro Met Ile Pro Cys Asn Glu Ser Cys Ser Val Val Gly
210 215 220

Lys Val Ile Ala Ser Gln Trp Pro Glu Glu Thr Phe Gly
225 230 235

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 91 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Val Arg Trp Thr Leu Trp Asp Thr Leu Ala Phe Leu Leu Leu Leu
1 5 10 15

Ser Leu Leu Leu Pro Ser Leu Leu Ile Met Phe Ile Pro Ser Thr Phe
20 25 30

Lys Arg Pro Val Ser Ser Trp Lys Ala Leu Asn Leu Arg Lys Thr Leu
35 40 45

Leu Met Ala Ser Ser Val Arg Leu Lys Pro Leu Asn Cys Ser Arg Leu
50 55 60

Pro Cys Val Tyr Ala Gln Glu Thr Leu Thr Phe Leu Leu Thr Gln Lys
65 70 75 80

Lys Thr Cys Val Lys Asn Tyr Val Gln Lys Glu
85 90

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2834 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: double strand
(D) TOPOLOGY: both

(vi) INITIAL ORIGIN:
(A) ORGANISM: pCSJ fragment

(xi) CHARACTERISTICS:
(A) NAME/KEY: CDS
(B) LOCATION: complement (106..816)

(ix) CHARACTERISTICS:
(A) NAME/KEY: CDS
(B) LOCATION: 1025..2104

(ix) CHARACTERISTICS:
(A) NAME/KEY: CDS
(B) LOCATION: 2377..2649

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```
ATTTACTATG TTATGTTCTG AGGGGAGTGA AAATTCCCCT AATTCGATGA AGATTCTTGC      60
TCAATTGTTA TCAGCTATGC GCCGACCAGA ACACCTTGCC GATCAGCCAA ACGTCTCTTC     120
AGGCCACTGA CTAGCGATAA CTTTCCCCAC AACGGAACAA CTCTCATTGC ATGGGATCAT     180
TGGGTACTGT GGGTTTAGTG GTTGTAAAAA CACCTGACCG CTATCCCTGA TCAGTTTCTT     240
GAAGGTAAAC TCATCACCCC CAAGTCTGGC TATGCAGAAA TCACCTGGCT CAACAGCCTG     300
CTCAGGGTCA ACGAGAATTA ACATTCCGTC AGGAAAGCTT GGCTTGGAGC CTGTTGGTGC     360
GGTCATGGAA TTACCTTCAA CCTCAAGCCA GAATGCAGAA TCACTGGCTT TTTTGGTTGT     420
GCTTACCCAT CTCTCCGCAT CACCTTTGGT AAAGGTTCTA AGCTTAGGTG AGAACATCCC     480
TGCCTGAACA TGAGAAAAAA CAGGGTACTC ATACTCACTT CTAAGTGACG GCTGCATACT     540
AACCGCTTCA TACATCTCGT AGATTTCTCT GGCGATTGAA GGGCTAAATT CTTCAACGCT     600
AACTTTGAGA ATTTTTGTAA GCAATGCGGC GTTATAAGCA TTTAATGCAT TGATGCCATT     660
AAATAAAGCA CCAACGCCTG ACTGCCCCAT CCCCATCTTG TCTGCGACAG ATTCCTGGGA     720
TAAGCCAAGT TCATTTTTCT TTTTTTCATA AATTGCTTTA AGGCGACGTG CGTCCTCAAG     780
CTGCTCTTGT GTTAATGGTT TCTTTTTTGT GCTCATACGT TAAATCTATC ACCGCAAGGG     840
ATAAATATCT AACACCGCGC GTGTTGACTA TTTTACCTCT GGCGGTGATA ATGGTTGCAT     900
GTACTAAGTA GGTTGTATGG AACAACGCAT AACCCTGAAA GATTATGCAA TGCGCTTTGG     960
GCAAACCAAG ACAGCTAAAG ATCCTCTAGA GCGCCCGGAA GAGAGTCAAT TCAGGGTGGT    1020
GAAT GTG AAA CCA GTA ACG TTA TAC GAT GTC GCA GAG TAT GCC GGT GTC      1069
     Val Lys Pro Val Thr Leu Tyr Asp Val Ala Glu Tyr Ala Gly Val
                  95                  100                 105

TCT TAT CAG ACC GTT TCC CGC GTG GTG AAC CAG GCC AGC CAC GTT TCT      1117
Ser Tyr Gln Thr Val Ser Arg Val Val Asn Gln Ala Ser His Val Ser
            110                 115                 120
```

```
GCG AAA ACG CGG GAA AAA GTG GAA GCG GCG ATG GCG GAG CTG AAT TAC      1165
Ala Lys Thr Arg Glu Lys Val Glu Ala Ala Met Ala Glu Leu Asn Tyr
        125                 130                 135

ATT CCC AAC CGC GTG GCA CAA CAA CTG GCG GGC AAA CAG TCG TTG CTG      1213
Ile Pro Asn Arg Val Ala Gln Gln Leu Ala Gly Lys Gln Ser Leu Leu
    140                 145                 150

ATT GGC GTT GCC ACC TCC AGT CTG GCC CTG CAC GCG CCG TCG CAA ATT      1261
Ile Gly Val Ala Thr Ser Ser Leu Ala Leu His Ala Pro Ser Gln Ile
155                 160                 165                 170

GTC GCG GCG ATT AAA TCT CGC GCC GAT CAA CTG GGT GCC AGC GTG GTG      1309
Val Ala Ala Ile Lys Ser Arg Ala Asp Gln Leu Gly Ala Ser Val Val
                175                 180                 185

GTG TCG ATG GTA GAA CGA AGC GGC GTC GAA GCC TGT AAA GCG GCG GTG      1357
Val Ser Met Val Glu Arg Ser Gly Val Glu Ala Cys Lys Ala Ala Val
            190                 195                 200

CAC AAT CTT CTC GCG CAA CGC GTC AGT GGG CTG ATC ATT AAC TAT CCG      1405
His Asn Leu Leu Ala Gln Arg Val Ser Gly Leu Ile Ile Asn Tyr Pro
        205                 210                 215

CTG GAT GAC CAG GAT GCC ATT GCT GTG GAA GCT GCC TGC ACT AAT GTT      1453
Leu Asp Asp Gln Asp Ala Ile Ala Val Glu Ala Ala Cys Thr Asn Val
    220                 225                 230

CCG GCG TTA TTT CTT GAT GTC TCT GAC CAG ACA CCC ATC AAC AGT ATT      1501
Pro Ala Leu Phe Leu Asp Val Ser Asp Gln Thr Pro Ile Asn Ser Ile
235                 240                 245                 250

ATT TTC TCC CAT GAA GAC GGT ACG CGA CTG GGC GTG GAG CAT CTG GTC      1549
Ile Phe Ser His Glu Asp Gly Thr Arg Leu Gly Val Glu His Leu Val
                255                 260                 265

GCA TTG GGT CAC CAG CAA ATC GCG CTG TTA GCG GGC CCA TTA AGT TCT      1597
Ala Leu Gly His Gln Gln Ile Ala Leu Leu Ala Gly Pro Leu Ser Ser
            270                 275                 280

GTC TCG GCG CGT CTG CGT CTG GCT GGC TGG CAT AAA TAT CTC ACT CGC      1645
Val Ser Ala Arg Leu Arg Leu Ala Gly Trp His Lys Tyr Leu Thr Arg
        285                 290                 295

AAT CAA ATT CAG CCG ATA GCG GAA CGG GAA GGC GAC TGG AGT GCC ATG      1693
Asn Gln Ile Gln Pro Ile Ala Glu Arg Glu Gly Asp Trp Ser Ala Met
    300                 305                 310

TCC GGT TTT CAA CAA ACC ATG CAA ATG CTG AAT GAG GGC ATC GTT CCC      1741
Ser Gly Phe Gln Gln Thr Met Gln Met Leu Asn Glu Gly Ile Val Pro
315                 320                 325                 330

ACT GCG ATG CTG GTT GCC AAC GAT CAG ATG GCG CTG GGC GCA ATG CGC      1789
Thr Ala Met Leu Val Ala Asn Asp Gln Met Ala Leu Gly Ala Met Arg
                335                 340                 345

GCC ATT ACC GAG TCC GGG CTG CGC GTT GGT GCG GAT ATC TCG GTA GTG      1837
Ala Ile Thr Glu Ser Gly Leu Arg Val Gly Ala Asp Ile Ser Val Val
            350                 355                 360

GGA TAC GAC GAT ACC GAA GAC AGC TCA TGT TAT ATC CCG CCG TCA ACC      1885
Gly Tyr Asp Asp Thr Glu Asp Ser Ser Cys Tyr Ile Pro Pro Ser Thr
        365                 370                 375

ACC ATC AAA CAG GAT TTT CGC CTG CTG GGG CAA ACC AGC GTG GAC CGC      1933
Thr Ile Lys Gln Asp Phe Arg Leu Leu Gly Gln Thr Ser Val Asp Arg
    380                 385                 390
```

```
TTG CTG CAA CTC TCT CAG GGC CAG GCG GTG AAG GGC AAT CAG CTG TTG      1981
Leu Leu Gln Leu Ser Gln Gly Gln Ala Val Lys Gly Asn Gln Leu Leu
395                 400                 405                 410

CCC GTC TCA CTG GTG AAA AGA AAA ACC ACC CTG GCG CCC AAT ACG CAA      2029
Pro Val Ser Leu Val Lys Arg Lys Thr Thr Leu Ala Pro Asn Thr Gln
                415                 420                 425

ACC GCC TCT CCC CGC GCG TTG GCC GAT TCA TTA ATG CAG CTG GCA CGA      2077
Thr Ala Ser Pro Arg Ala Leu Ala Asp Ser Leu Met Gln Leu Ala Arg
            430                 435                 440

CAG GTT TCC CGA CTG GAA AGC GGG CAG TGAGCGCAAC GCAATTAATG            2124
Gln Val Ser Arg Leu Glu Ser Gly Gln
        445                 450

TGAGTTAGCT CACTCATTAG GCACCCCAGG CTTTACACTT TATGCTTCCG GCTCGTATGT    2184

TGTGTGGAAT TGTGAGCGGA TAACAATTTC ACACAGGAAA CAGCTCTGCA GGCATGCAAG    2244

CTTATCGAAT TCTCATTCAG GCTTCTGCCG TTTTGGATTT AACCGAAGAT GATTTCGATT    2304

TTCTGACGAG TAACAAAGTT TGGATTGCTA CTGACCGCTC TCGTGCTCGT CGCTGCGTTG    2364

AGGCTTGCGT TT ATG GTA CGC TGG ACT TTG TGG GAT ACC CTC GCT TTC        2412
              Met Val Arg Trp Thr Leu Trp Asp Thr Leu Ala Phe
                1               5                   10

CTG CTC CTG TTG AGT TTA TTG CTG CCG TCA TTG CTT ATT ATG TTC ATC      2460
Leu Leu Leu Leu Ser Leu Leu Leu Pro Ser Leu Leu Ile Met Phe Ile
          15                  20                  25

CCG TCA ACA TTC AAA CGG CCT GTC TCA TCA TGG AAG GCG CTG AAT TTA      2508
Pro Ser Thr Phe Lys Arg Pro Val Ser Ser Trp Lys Ala Leu Asn Leu
     30                  35                  40

CGG AAA ACA TTA TTA ATG GCG TCG AGC GTC CGG TTA AAG CCG CTG AAT      2556
Arg Lys Thr Leu Leu Met Ala Ser Ser Val Arg Leu Lys Pro Leu Asn
 45                  50                  55                  60

TGT TCG CGT TTA CCT TGC GTG TAC GCG CAG GAA ACA CTG ACG TTC TTA      2604
Cys Ser Arg Leu Pro Cys Val Tyr Ala Gln Glu Thr Leu Thr Phe Leu
                65                  70                  75

CTG ACG CAG AAG AAA ACG TGC GTC AAA AAT TAC GTG CAG AAG GAG          2649
Leu Thr Gln Lys Lys Thr Cys Val Lys Asn Tyr Val Gln Lys Glu
            80                  85                  90

TGATGTAATG TCTAAAGGTA AAAAACGTTC TGGCGCTCGC CCTGGTCGTC CGCAGCCGTT    2709

GCGAGGTACT AAAGGCAAGC GTAAAGGCGC TCGTCTTTGG TATGTAGGTG GTCAACAATT    2769

TTAATTGCAG GGGCTTCGGC CCTTACTTGA GGATAAATTA TGTCTAATAT TCAAACTGGC    2829

GCCGA                                                               2834
```

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 237 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Met Ser Thr Lys Lys Lys Pro Leu Thr Gln Glu Gln Leu Glu Asp Ala
 1               5                  10                  15

Arg Arg Leu Lys Ala Ile Tyr Glu Lys Lys Lys Asn Glu Leu Gly Leu
            20                  25                  30

Ser Gln Glu Ser Val Ala Asp Lys Met Gly Met Gly Gln Ser Gly Val
        35                  40                  45

Gly Ala Leu Phe Asn Gly Ile Asn Ala Leu Asn Ala Tyr Asn Ala Ala
    50                  55                  60

Leu Leu Thr Lys Ile Leu Lys Val Ser Val Glu Glu Phe Ser Pro Ser
65                  70                  75                  80

Ile Ala Arg Glu Ile Tyr Glu Met Tyr Glu Ala Val Ser Met Gln Pro
                85                  90                  95

Ser Leu Arg Ser Glu Tyr Glu Tyr Pro Val Phe Ser His Val Gln Ala
            100                 105                 110

Gly Met Phe Ser Pro Lys Leu Arg Thr Phe Thr Lys Gly Asp Ala Glu
        115                 120                 125

Arg Trp Val Ser Thr Thr Lys Lys Ala Ser Asp Ser Ala Phe Trp Leu
    130                 135                 140

Glu Val Glu Gly Asn Ser Met Thr Ala Pro Thr Gly Ser Lys Pro Ser
145                 150                 155                 160

Phe Pro Asp Gly Met Leu Ile Leu Val Asp Pro Glu Gln Ala Val Glu
                165                 170                 175

Pro Gly Asp Phe Cys Ile Ala Arg Leu Gly Gly Asp Glu Phe Thr Phe
            180                 185                 190

Lys Lys Leu Ile Arg Asp Ser Gly Gln Val Phe Leu Gln Pro Leu Asn
        195                 200                 205

Pro Gln Tyr Pro Met Ile Pro Cys Asn Glu Ser Cys Ser Val Val Gly
    210                 215                 220

Lys Val Ile Ala Ser Gln Trp Pro Glu Glu Thr Phe Gly
225                 230                 235
```

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 360 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

```
Val Lys Pro Val Thr Leu Tyr Asp Val Ala Glu Tyr Ala Gly Val Ser
 1               5                  10                  15

Tyr Gln Thr Val Ser Arg Val Val Asn Gln Ala Ser His Val Ser Ala
            20                  25                  30

Lys Thr Arg Glu Lys Val Glu Ala Ala Met Ala Glu Leu Asn Tyr Ile
        35                  40                  45

Pro Asn Arg Val Ala Gln Gln Leu Ala Gly Lys Gln Ser Leu Leu Ile
    50                  55                  60

Gly Val Ala Thr Ser Ser Leu Ala Leu His Ala Pro Ser Gln Ile Val
65                  70                  75                  80

Ala Ala Ile Lys Ser Arg Ala Asp Gln Leu Gly Ala Ser Val Val Val
                85                  90                  95

Ser Met Val Glu Arg Ser Gly Val Glu Ala Cys Lys Ala Ala Val His
            100                 105                 110

Asn Leu Leu Ala Gln Arg Val Ser Gly Leu Ile Ile Asn Tyr Pro Leu
        115                 120                 125

Asp Asp Gln Asp Ala Ile Ala Val Glu Ala Ala Cys Thr Asn Val Pro
    130                 135                 140

Ala Leu Phe Leu Asp Val Ser Asp Gln Thr Pro Ile Asn Ser Ile Ile
145                 150                 155                 160

Phe Ser His Glu Asp Gly Thr Arg Leu Gly Val Glu His Leu Val Ala
                165                 170                 175

Leu Gly His Gln Gln Ile Ala Leu Leu Ala Gly Pro Leu Ser Ser Val
            180                 185                 190

Ser Ala Arg Leu Arg Leu Ala Gly Trp His Lys Tyr Leu Thr Arg Asn
        195                 200                 205

Gln Ile Gln Pro Ile Ala Glu Arg Glu Gly Asp Trp Ser Ala Met Ser
    210                 215                 220

Gly Phe Gln Gln Thr Met Gln Met Leu Asn Glu Gly Ile Val Pro Thr
225                 230                 235                 240

Ala Met Leu Val Ala Asn Asp Gln Met Ala Leu Gly Ala Met Arg Ala
                245                 250                 255

Ile Thr Glu Ser Gly Leu Arg Val Gly Ala Asp Ile Ser Val Val Gly
            260                 265                 270

Tyr Asp Asp Thr Glu Asp Ser Ser Cys Tyr Ile Pro Pro Ser Thr Thr
        275                 280                 285

Ile Lys Gln Asp Phe Arg Leu Leu Gly Gln Thr Ser Val Asp Arg Leu
    290                 295                 300

Leu Gln Leu Ser Gln Gly Gln Ala Val Lys Gly Asn Gln Leu Leu Pro
305                 310                 315                 320

Val Ser Leu Val Lys Arg Lys Thr Thr Leu Ala Pro Asn Thr Gln Thr
                325                 330                 335
```

Ala Ser Pro Arg Ala Leu Ala Asp Ser Leu Met Gln Leu Ala Arg Gln
340 345 350

Val Ser Arg Leu Glu Ser Gly Gln
355 360

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 91 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Met Val Arg Trp Thr Leu Trp Asp Thr Leu Ala Phe Leu Leu Leu Leu
1 5 10 15

Ser Leu Leu Leu Pro Ser Leu Leu Ile Met Phe Ile Pro Ser Thr Phe
20 25 30

Lys Arg Pro Val Ser Ser Trp Lys Ala Leu Asn Leu Arg Lys Thr Leu
35 40 45

Leu Met Ala Ser Ser Val Arg Leu Lys Pro Leu Asn Cys Ser Arg Leu
50 55 60

Pro Cys Val Tyr Ala Gln Glu Thr Leu Thr Phe Leu Leu Thr Gln Lys
65 70 75 80

Lys Thr Cys Val Lys Asn Tyr Val Gln Lys Glu
85 90

Claims

1. Method for selecting mutated O_R or O_L operator DNA sequences from lambdoid phages which have a different thermostability compared to the wild-type sequence with regard to binding a repressor,
wherein
(a) a DNA cassette is prepared which contains a selection gene under the operative control of an expression control sequence comprising at least one O_R or O_L operator sequence from a lambdoid phage and a promoter,
(b) the operator DNA sequence is subjected to a mutagenesis and
(c) the mutated operator DNA sequences are analysed.

2. Method as claimed in claim 1,
wherein
the lambdoid phages are selected from the group comprising the phage lambda, phage 21, phage 22, phage 82, phage 424, phage 434, phage D326, phage DLP12, phage gamma, phage HK022, phage P4, phage Phi80, phage Phi81, coliphage 186 and recombinant variants thereof.

3. Method as claimed in claim 2,
wherein
the phage lambda or recombinant variants thereof are used.

4. Method as claimed in claim 3,
wherein
an operator DNA sequence from the operator regions O_R or/and O_L of the phage lambda is used.

5. Method as claimed in one of the claims 1 - 4,
wherein
the E-lysis gene from the phage PhiX174 is used as the selection gene.

6. Method as claimed in one of the claims 1 - 5,
wherein
the operator DNA sequence is subjected to a site-specific mutagenesis by oligonucleotides or a selection is carried out in a mutator bacterial strain.

7. Method as claimed in one of the claims 1 - 6,
wherein
the mutated operator DNA sequences are analysed by determining their ability to bind to a temperature-sensitive cI repressor.

8. Method as claimed in claim 7,
wherein
the temperature-sensitive lambda repressor cI857 is used.

9. Mutated O_R or O_L operator sequences from lambdoid phages which have a different thermostability compared to the wild-type sequence with regard to binding of a repressor and are obtainable by a method as claimed in one of the claims 1 - 8.

10. Mutated O_R or O_L operator sequences from lambdoid phages which have an increased thermostability compared to the wild-type sequence with regard to binding of a temperature-sensitive repressor and are obtainable by a method as claimed in one of the claims 1 - 8.

11. Mutated O_R or O_L operator sequence as claimed in claim 10,
wherein
it has an approximately 3 - 10°C increased thermostability.

12. Mutated O_R or O_L operator sequence as claimed in claim 10,
wherein
it has an approximately 7 - 9°C increased thermostability.

13. Mutated lambda O_R or O_L operator sequence as claimed in one of the claims 9 - 12, which is a variant of the sequences shown in SEQ ID NO.1 or SEQ ID NO.3.

14. Mutated lambda O_R operator sequence comprising the sequence shown in SEQ ID NO.2.

15. Use of a mutated O_R or O_L operator sequence as claimed in one of the claims 9 - 14 for the temperature-regulated expression of genes in bacterial cells.

16. Use of a combination of (a) a wild-type O_R or O_L operator region and at least one operator region which contains a mutated O_R or O_L operator sequence as claimed in one of the claims 9 - 14 or (b) several operator regions which contain mutated O_R or O_L operator sequences as claimed in one of the claims 9 - 14 with different thermostabilities for the temperature-regulated sequential expression of genes.

17. Use as claimed in claim 15 or 16,
wherein
the bacterial cells contain a gene for a cI repressor from lambdoid phages for the regulation of gene expression.

18. Use as claimed in claim 17,
wherein
the bacterial cells contain the gene for the lambda cI857 repressor.

19. Nucleic acid comprising a bacterial expression control sequence which contains a mutated O_R or O_L operator sequence as claimed in one of the claims 9 - 14 in operative linkage with a protein-coding sequence.

20. Nucleic acid as claimed in claim 19,
wherein
the protein-coding sequence is a suicide gene.

21. Nucleic acid as claimed in claim 20,
wherein
the expression control sequence contains a lambda P_L or P_R promoter.

22. Vector,
wherein
it contains at least one copy of a nucleic acid as claimed in one of the claims 19 - 21.

23. Vector as claimed in claim 22,
wherein
it is a bacterial chromosomal vector.

24. Vector as claimed in claim 22,
wherein
it is a bacterial extrachromosomal plasmid.

25. Bacterial cell,
wherein
it is transformed with a nucleic acid as claimed in one of the claims 19 - 21 or with a vector as claimed in one of the claims 22 - 24.

26. Bacterial cell as claimed in claim 25,
wherein
it contains the nucleic acid or the vector integrated into its chromosome.

27. Bacterial cell as claimed in claim 25 or 26,
wherein
it additionally contains a gene for a cI repressor from lambdoid phages.

28. Bacterial cell as claimed in claim 27,
wherein
it contains the gene for the lambda cI857 repressor.

29. Vaccine composition,
wherein
it contains a live bacterial cell as claimed in one of the claims 26 - 28 as an active ingredient optionally with pharmaceutically acceptable auxiliary substances, additives and carrier substances.

30. Vaccine composition,
wherein
it contains a bacterial ghost as the active ingredient optionally with pharmaceutically acceptable auxiliary substances, additives and carrier substances in which the bacterial ghost can be obtained by culturing a bacterial cell as claimed in one of the claims 25 - 28 at temperatures of 35 - 39°C and subsequently lysing the bacterial cell by increasing the temperature.

31. Nucleic acid comprising (a) a first bacterial expression control sequence which contains an O_R or O_L operator sequence from a lambdoid phage and to which a first cI repressor from lambdoid phages can bind, in operative linkage with a sequence coding for a second repressor wherein the second repressor cannot bind to the first bacterial expression sequence and (b) a second bacterial expression control sequence to which the second repressor can bind in operative linkage with a suicide gene.

32. Bacterial cell,
wherein
it contains at least one copy of a nucleic acid as claimed in claim 31.

33. Bacterial cell as claimed in claim 32,
wherein
it additionally contains a gene for the first repressor.

34. Bacterial cell as claimed in claim 32 or 33,
wherein
it contains the first bacterial expression control sequence of a mutated operator sequence as claimed in one of the claims 9 - 14.

35. Bacterial cell as claimed in one of the claims 32 - 34 additionally comprising (c) a third bacterial expression control sequence which contains a mutated operator sequence as claimed in one of the claims 9 - 14 in operative linkage with a suicide gene.

36. Vaccine composition,
wherein
it contains a live bacterial cell as claimed in one of the claims 32 - 35 as the active ingredient optionally together with pharmaceutically acceptable auxiliary substances, additives and carrier substances.

37. Use of vaccine compositions as claimed in claim 29 or 36 as heat-sensitive or/and cold-sensitive safe live vaccines.

PCT WELTORGANISATION FÜR GEISTIGES EIGENTUM
Internationales Büro
INTERNATIONALE ANMELDUNG VERÖFFENTLICHT NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT)

(51) Internationale Patentklassifikation 6 : C12N 15/73, 15/10, A61K 39/02 // C 12N 9/36 | A3

(11) Internationale Veröffentlichungsnummer: WO 98/07874

(43) Internationales Veröffentlichungsdatum: 26. Februar 1998 (26.02.98)

(21) Internationales Aktenzeichen: PCT/EP97/04560

(22) Internationales Anmeldedatum: 21. August 1997 (21.08.97)

(30) Prioritätsdaten:
196 33 698.8 21. August 1996 (21.08.96) DE

(71)(72) Anmelder und Erfinder: LUBITZ, Werner [AT/AT]; Schönborngasse 12/7, A-1080 Wien (AT).

(72) Erfinder; und

(75) Erfinder/Anmelder *(nur für US)*: JECHLINGER, Wolfgang [AT/AT]; Strozzigasse 38/12, A-1080 Wien (AT). SZOSTAK, Michael [AT/AT]; In den Schnablern 9/3, A-2344 Maria Enzersdorf (AT).

(74) Anwälte: WEICKMANN, H. usw.; Kopernikusstrasse 9, D-81679 München (DE).

(81) Bestimmungsstaaten: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO Patent (GH, KE, LS, MW, SD, SZ, UG, ZW), eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches Patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Veröffentlicht
Mit internationalem Recherchenbericht.
Vor Ablauf der für Änderungen der Ansprüche zugelassenen Frist. Veröffentlichung wird wiederholt falls Änderungen eintreffen.

(88) Veröffentlichungsdatum des internationalen Recherchenberichts: 26. März 1998 (26.03.98)

(54) Title: NEW SYSTEMS FOR REGULATING GENETIC EXPRESSION

(54) Bezeichnung: NEUE SYSTEME ZUR REGULATION DER GENEXPRESSION

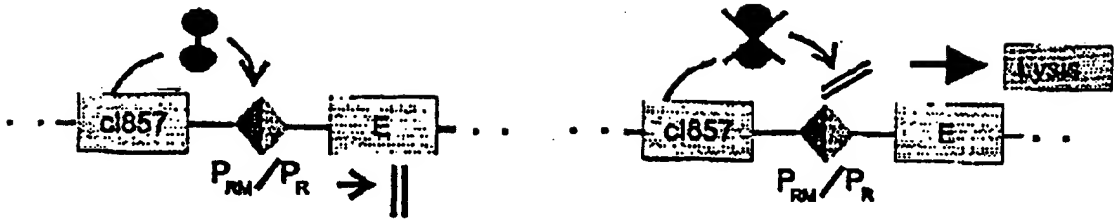
(57) Abstract

The present invention concerns a process for selecting new P_R- or P_L-operator sequences of lambdoid phages which, compared to wild-type sequences, have a different thermostability for the binding of a repressor. In addition, the invention discloses new mutated PR- or PL- operator sequences and their use for temperature-regulated expression of genes and for producing improved vaccines.

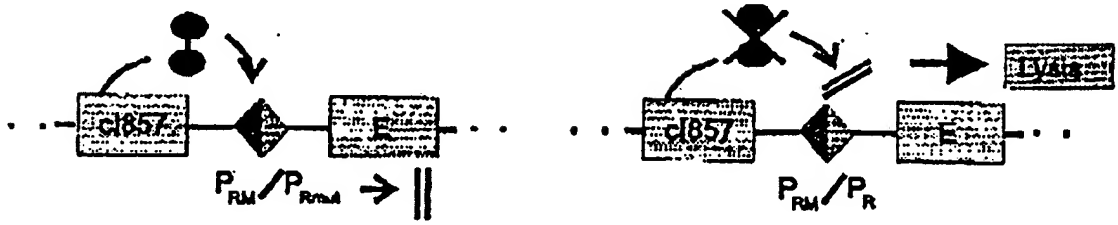
(57) Zusammenfassung

Die vorliegende Erfindung betrifft ein Verfahren zur Sclektion neuer P_R- oder P_L-Operatorsequenzen aus lambdoiden Phagen, die eine im Vergleich zur Wildtypsequenz unterschiedliche Thermostabilität hinsichtlich der Bindung eines Repressors aufweisen. Weiterhin werden neue mutierte P_R- oder P_L-Operatorsequenzen sowie deren Verwendung zur temperaturregulierten Expression von Genen und zur Herstellung verbesserter Impfstoffe offenbart.

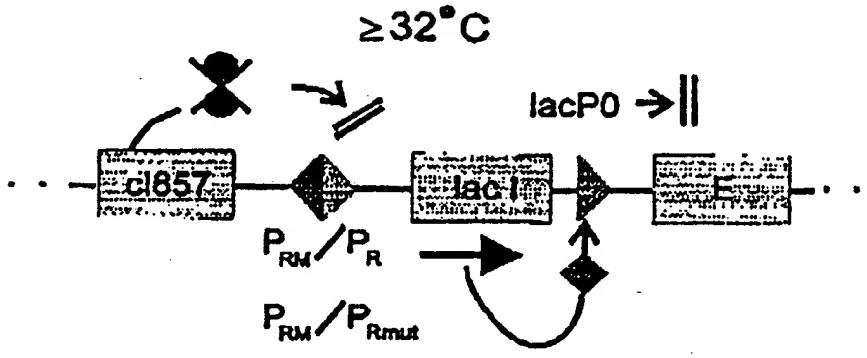
1a) pAW12 ≤30°C



b) pAWJ12 ≤37°C



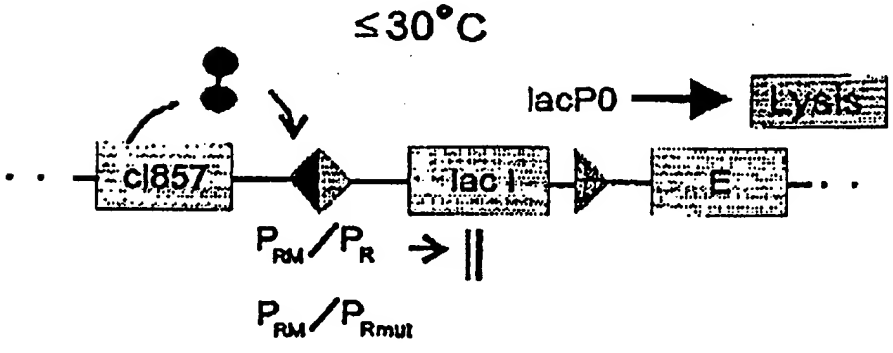
2a)



pCS1

pCSJ1

2b)



pCS1

pCSJ1

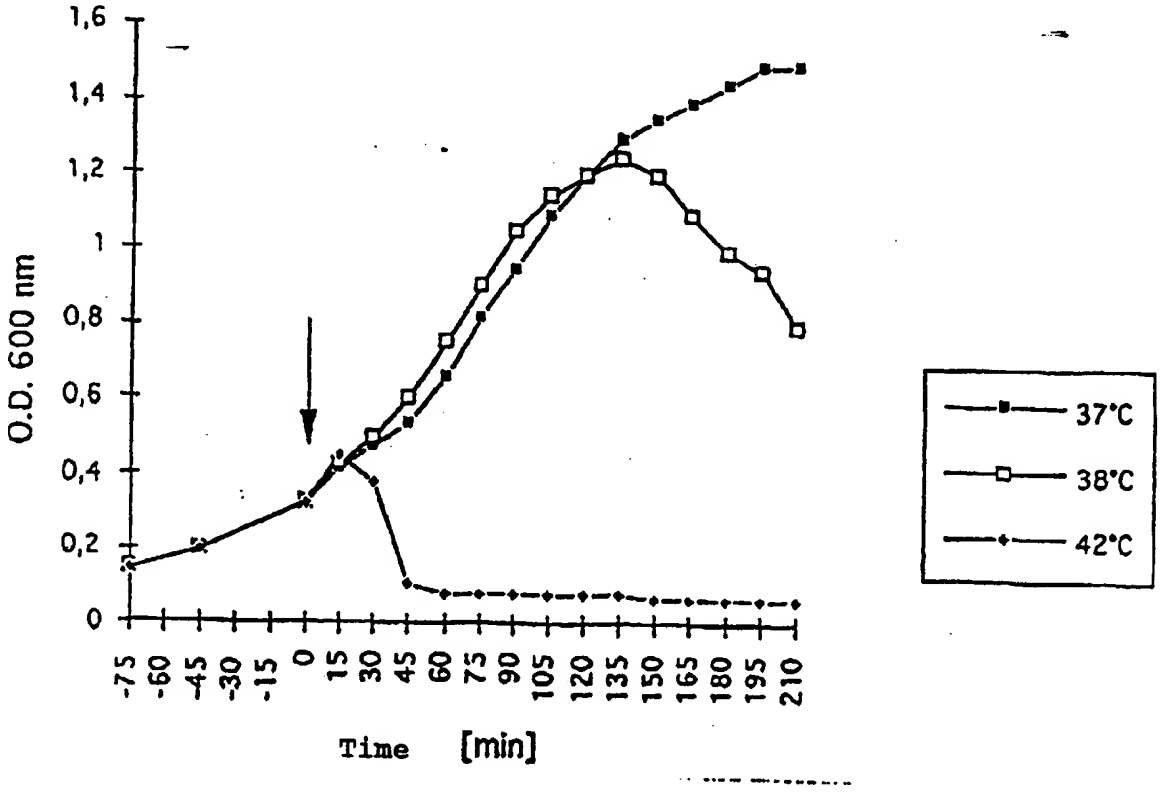


Fig. 3: Growth of E. coli NM522 (pAWJ12) when the temperature is changed from 28°C to higher temperatures (↓)

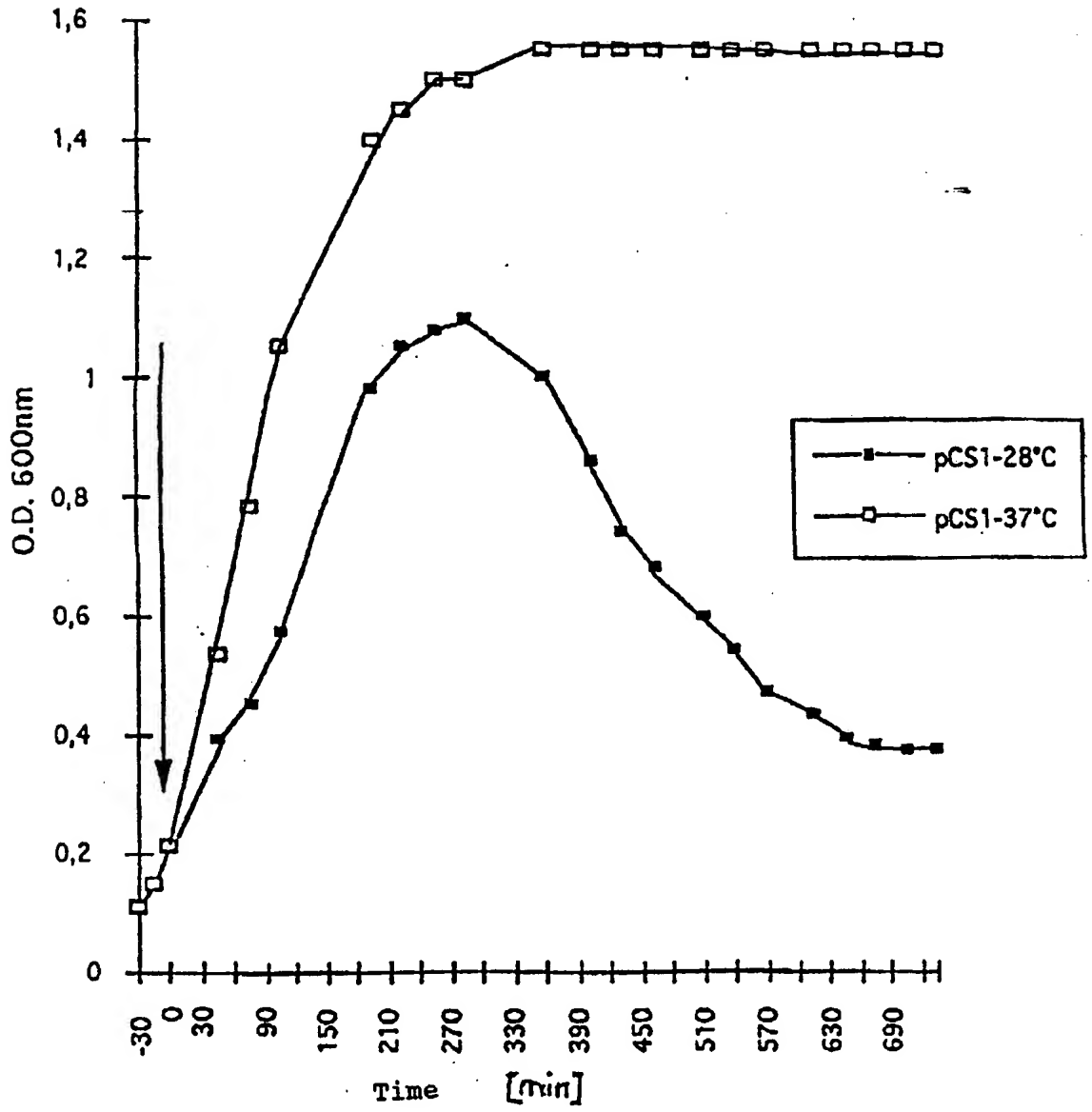


Fig. 4: Growth of E. coli MC4100 (pCS1) when the temperature is changed from 37°C to 28°C (↓)

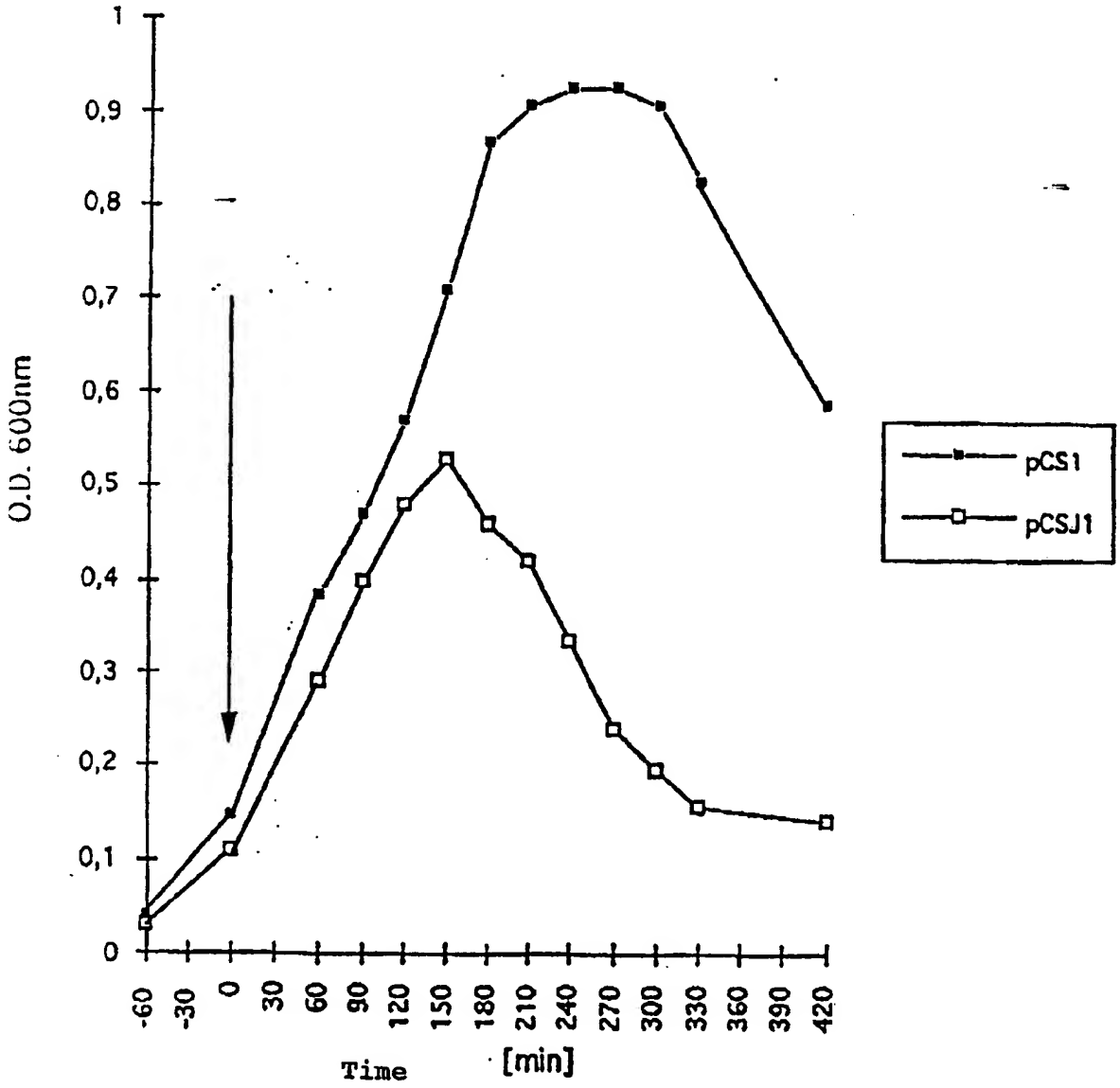


Fig. 5: Growth of E. coli MC4100 (pCS1) and MC4100 (pCSJ1) when the temperature is changed from 37°C to 28°C (↓)

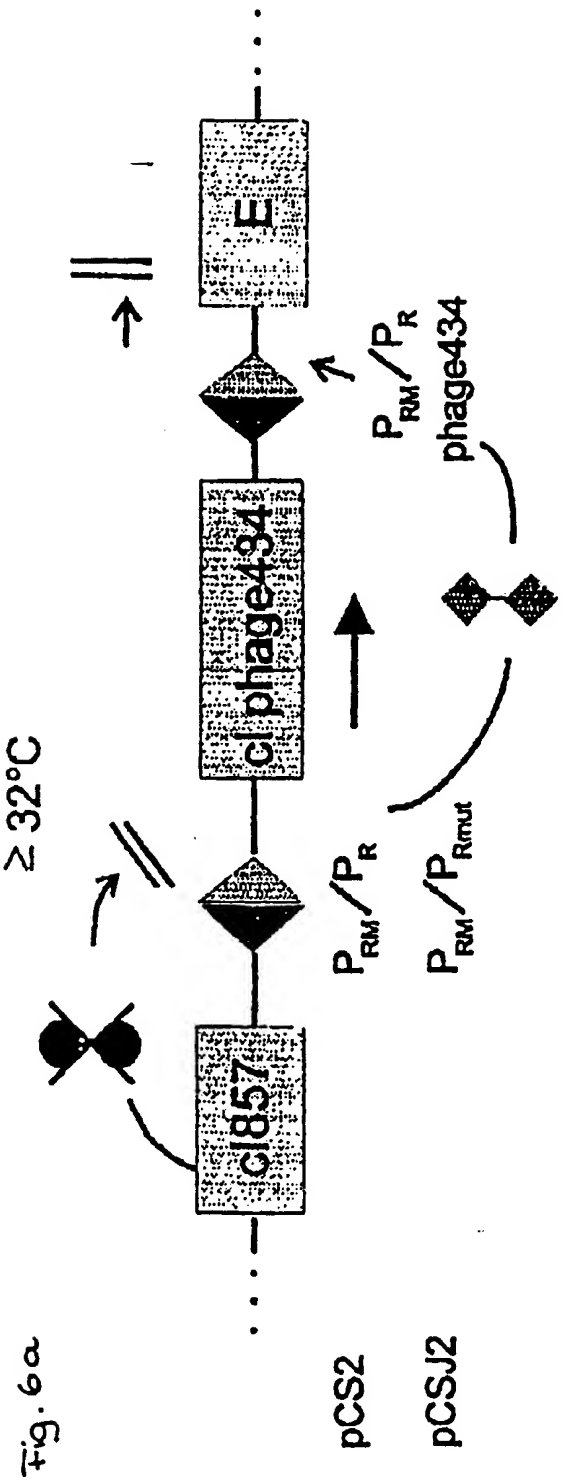

≥ 32°C
cI857
cI phage434
E
P_{RM}/P_R
P_{RM}/P_{Rmut}
phage434
pCS2
pCSJ2

Fig. 6a

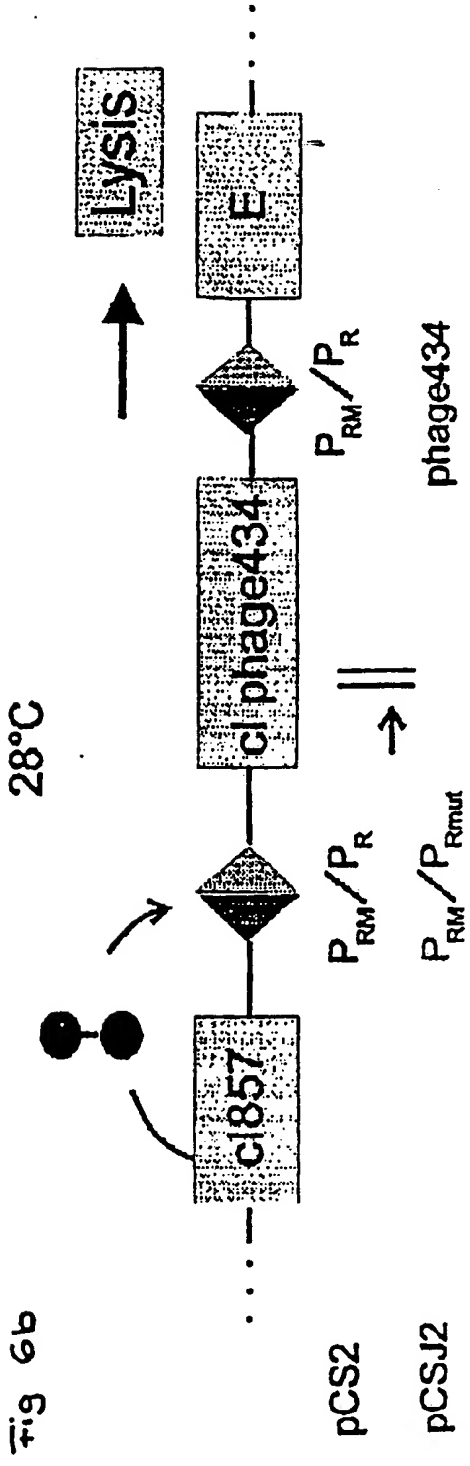

28°C
Lysis
cI857
cI phage434
E
P_{RM}/P_R
P_{RM}/P_{Rmut}
phage434
pCS2
pCSJ2

Fig 6b